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Process for the Detection of the Phenomenon of Fluorescence in a Microscope

The process that is presented relates to confocal and 2 photon fluorescence microscopy [Goeppert] [Wilson]. Both methods are assumed to be known.

Both confocal fluorescence microscopy and 2 photon microscopy are modified by the method presented below to the extent that an additional contrast parameter is possible: the fluorescence lifetime.

Temporal resolved fluorescence and / or the use of the lifetime as a contrast parameter in confocal / 2 photon microscopy can be carried out by two different methods - by time domain detection and frequency domain detection.

In the case of **time domain detection** [Wabnitz] [Gröbler] a fluorescent sample is excited so as to produce fluorescence by means of a pulsed light source, and the fluorescence emission is detected with time resolution either by means of time correlated single photon counting (TCSPC) [Müller] [Han] or by means of time gated detection [Schbg] [Cubeddu] [Dowling].

In the case of **frequency domain detection**, a fluorescent sample or preparation is excited with a light source that is either actively modulated or pulsed (for example, by means of passive mode coupling). Since any arbitrary modulation of the excitation by means of a Fourier analysis falls part into sinusoidal components, the observation of a sinusoidal excitation is adequate. The frequency domain detection technique is based on the delay of the fluorescence emission by a phase f and a change in the modulation depth M compared with the excitation light as a function of the modulation frequency ω ($=2 \pi f_{mod}$) and the lifetime τ.

$$\varphi = \text{atan}\,(\omega\,\tau) \tag{1}$$

$$M = 1 / \sqrt{(1+\omega^2\,\tau^2)} \tag{2}$$

However, the resulting fluorescence signal, oscillated with the modulation frequency, is out of phase and demodulated. For typical fluorescence lifetimes ranging from τ = 1...10 ns, modulation frequencies ranging from f_{mod} = 10...100 MHz are adequate.

Since it generally does not make any sense to scan the fluorescence signal at such high modulation frequencies, a frequency mixing process is used to detect the signal. For mixing, any detection element with a modulatable amplification is suitable.

In essence two methods are distinguished - the homodyne and the heterodyne detection techniques.

To understand the principle, one observes generally two "signals" S_1, S_2, (for example S_1 modulated excitation, S_2 modulated amplification).

$$S_1 = A_0 + A_1 \cos(\omega_a t + \alpha)$$
$$S_2 = B_0 + B_1 \cos(\omega_b t + \beta) \tag{3}$$

Multiplication results in:

$$S_1S_2 = A_0B_0 + A_0B_1 \cos(\omega_b t + \beta) + B_0A_1 \cos(\omega_a t + \alpha) + A_1B_1 \{\cos((\omega_a + \omega_b)t + (\alpha + \beta)) + \cos((\omega_a - \omega_b)t + (\alpha - \beta))\} \tag{4}$$

Homodyne Detection

If $\omega_a = \omega_b$ (homodyne), the second harmonic and a frequency independent component are generated by the mixing process of the "signals" S_1 and S_2. A low pass filter results in a suppression of the components at ω_a and $2\omega_a$. Only the

The DC background and the phase dependent DC component are detected. The signal, filtered by means of a low pass (LP = low pass), can be written as:

$$LP(S_1S_2) = A_0B_0 + A_1B_1 \cos(\alpha - \beta) \quad (5)$$

In the case of homodyne detection this frequency independent (DC) signal can be detected in multiple relative phases. To measure the fluorescence lifetime, at least 3 different phase positions are necessary. At just two relative phase positions, the phase shift or the demodulation, induced by the fluorescence lifetime, can be used as the contrast parameter (Clegg).

Heterodyne Detection - Cross Correlation

If $\omega_b = \omega_a + Dw$ (heterodyne), the mixing process generates a high frequency signal at the total frequency and a signal at the cross correlation frequency $\Delta\omega$. Again the high frequency components are suppressed by a low pass filter.

$$LP(S_1S_2) = A_0B_0 + A_1B_1 \cos(\Delta\omega t + \alpha - \beta) \quad (6)$$

In the case of heterodyne detection, the differential frequency $\Delta\omega$ is detected. Phase position and modulation depth of the signal at the differential frequency make it possible to determine the lifetime. Typical cross correlation frequencies range from a few Hz up to about 100 kHz.

A more comprehensive presentation of the heterodyne method can be found in the publication by E. Gratton et al. [Gratton] [Gratton 2].

In both the homodyne and heterodyne detection technique the high frequency change is reflected, so to say, on the low frequency range.

Owing to the multi-exponential decay behavior of the fluorescence emission, the lifetimes, determined from the modulation depth and the phase shift, vary. Therefore, for precise measurement of the lifetime, the excitation frequency has to be varied [Gratton2].

If, in contrast, the lifetime is supposed to be used, for example, as a contrast parameter in an imaging process, this is generally not necessary. Frequently it suffices, for example, to show the phase shift or the demodulation by means of the lifetime or by means of the lifetime, calculated from the phase shift and demodulation data, at a fixed modulation frequency.

1.1 Confocal Lifetime Imaging, Homodyne

The confocal design of an LSM [= laser scanning microscope] is modified for lifetime imaging to the extent that

- the excitation light source is modulated or a pulse laser is used.
- the amplification of the detector (for example, PMT) is modified.
- an electronic phase shifter is used that permits the relative phase position of light excitation and detection to be adjusted. This circuit with the components that are used is shown in Figure 1.

Realization 1:

Driven by means of a synthesizer S using a quartz crystal, RF frequencies are generated in the range from 10 to 100 MHz. The output of the synthesizer S is connected, on the one hand, over a shielded high frequency line (for example, over a BNC cable) to a phase shifter / amplifier PA. The phase shifter / amplifier amplifies, on the one hand, the high frequency input signal of the synthesizer - in the presented realization to a power of approx. 1.5 W at a resistance of 50W. Furthermore, the phase position of the amplified RF signal can be varied with the PA. The relative phase of the PA can be adjusted digitally over a control line, for example, by means of a control via a serial interface of a PC. The amplified RF signal is fed to a

modulated PMT [= photomultiplier tube] - for example, the PMT module (H 6573) from Hamamatsu.[1] The amplified RF voltage (approximately 25 V_{pp} / 50W) is fed in the PMT to the second dynode and used to modulate the amplification of the PMT. To produce a defined low pass filter, the output signal of the PMT can be smoothed by means of a commercial low pass (LP) filter. The 3 dB threshold frequency f_g of the LP filter is selected in such a manner that

$$1 / \text{pixel dwell time} << f_g << f_{laser}$$

where f_{laser} = the laser modulation frequency. The signal filtered thus is fed to the standard detection electronics of the LRM [= laser raster microscope]. (For greater comprehension: in principle, for example, to an ADC (analog digital converter), which is synchronized with the xy scanners of the LRM.

A second identical output of the synthesizer S is also connected to the control electronics of the light modulator M by means of a high frequency line. An acousto-optical modulator (or an electro-optical modulator) can be used, for example, as the modulator in the laser beam path. The light modulator is a component of the laser module of the confocal laser raster microscope. Such a design makes it possible to vary the relative phase position of the PA and the light modulator M. The frequencies for M for modulating the laser light and the PMT amplification voltage are identical. The phase position generally varies. At least the phase of an RF output can be adjusted digitally in about 1° steps. In the case depicted in Figure 1, the phase of the PMT modulation voltage is varied in PA by means of the phase shifter.

Description of the Operating Mode and the Related Advantages of the Method Presented
Using the conventional LSM design, the light of the modulated / pulsed laser light source is focussed on the lens plane by means of the scan mirrors and the LSM scan optics. The fluorescence is focussed, as in the confocal Zeiss LSM510,

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[1] The "Technical Information" to the H6573 from Hamamatsu contains a detailed presentation of the operating mode.

in the direction of reflection on the detection pin hole using a beam splitter, focussing lens, etc. The raster-shaped movement of the laser focus over the lens plane and the synchronized detection - in the now modulated - PMT result in a confocal image of the lens plane. By using the homodyne technique, the PMT signal at the output of the LP filter (image 1) is a DC signal, which varies only as a function of the laser spot position (pixel). Through repeated scanning operations during multiple different relative phases of the light modulator and detection modulation, it is possible to detect the fluorescence lifetime contrast or to measure the fluorescence lifetime. The significant advantage lies in the fact that the conventional data acquisition unit of the LRM can be used. The lifetime contrast is calculated from image to image.

Image Digitizing to Produce the Lifetime Contrast in a Confocal Microscope Image and / or to Display the Confocal Lifetime Distribution of Dyes.

- In a first step in the realization presented here, a first phase of the PMT detector is adjusted digitally. The adjustment is done, as described above, with the electronic PA phase shifter / amplifier; and the resulting DC signal of the PMT (following the usual conversion from current to voltage and the usual conversion from analog to digital) is registered by means of the usual (LSM) analysis electronics in synchronism with the scanner and stored in the PC by means of a storage medium.

  In at least a second step the process is repeated with a second different relative phase position.

The at least two digital images are displayed, for example, by means of a computer screen without any further calculation.

For the presentation, images, generated with the following algorithms, are also displayed on the screen (Fourier expansion):

The Fourier expansion is expressed by

$$I(\varphi + \varphi_\tau) = a_0 + a_1 \bullet \sin(\varphi) + b_1 \bullet \cos(\varphi) \qquad (7)$$

With the fluorescence intensity of a pixel I and the corresponding Fourier coefficients a_0, a_1, b_1.

$$a_0 = \bar{I} = \frac{1}{N} \sum_{M=0}^{M-1} I\left(\varphi_\tau + n \frac{2\pi}{N}\right)$$

$$a_1 = \frac{2}{N} \sum_{n=0}^{n-1} \sin\left(\frac{2\pi n}{N}\right) I\left(\varphi_\tau + n \frac{2\pi}{N}\right) \qquad (8)$$

$$b_1 = \frac{2}{N} \sum_{M=0}^{M-1} \cos\left(\frac{2\pi n}{N}\right) I\left(\varphi_\tau + n \frac{2\pi}{N}\right)$$

At the same time a_0, a_1, b_0 are the Fourier coefficients (per pixel); N > = 2 the number of the stored phase images (or pixel intensity) /=/ (φ).

The modulation depth M_τ and the phase shift can be expressed by the lifetime φ_τ using the Fourier coefficients.

$$M_\tau = \frac{\sqrt{(a_1^2 + b_1^2)}}{a_0} = \frac{1}{\sqrt{(1 + \omega^2 \tau^2)}} \qquad (9)$$

$$\varphi_\tau = -1 \bullet \frac{a_1}{b_1} = \omega\tau \qquad (10)$$

The modulation depth M is calculated pixel by pixel. M = M (i,j) = M_U, (i,j, pixel indices). Similarly the phase shift $\varphi = \varphi(i,j) = \varphi_U$.

The image of the modulation depth M_U, which is calculated pixel by pixel in this manner, and the phase shift φ_U, are displayed on the monitor.

Another type of display is the τ imaging, that is the lifetime $\tau(M)_{i,j}$ or $\tau(\varphi)_{i,j}$, calculated for τ by means of resolution equations (9) or (10) [Clegg].

$$\tau(M) = 1/\omega \quad \sqrt{[(1 / (M^2) - 1]} \tag{11}$$

$$\tau(\omega) = \frac{1}{\omega} \tan(\varphi) \tag{12}$$

M and φ are calculated with the equation (9) or (10); and the Fourier coefficients a_0, a_1, b_1, determined with equation (8), are calculated.

To increase the accuracy in determining the phase, it is also possible to use the optimized algorithm, presented in a paper, Phase Evaluation by Folding, by Küchel, 1989.

Realization 2:
Another realization of a confocal microscope with lifetime contrast or for the measurement of the lifetime distribution in a confocal split image can be achieved using pulse lasers. Suitable are, for example, pulse laser diodes or other, for example fs [= femtosecond] laser systems (Ti:saphire laser), for example with downstream frequency conversion unit (for example, frequency doubling, tripling). When pulse lasers are used, there is no need to generate the RF driver frequency with synthesizers. Instead, an electronic diode signal of the pulse laser is provided (for example, PD signal out of the fs / ps NIR laser in Figure 1 or an equivalent construction with exchange / expansion of the VIS laser module, for example with a ps diode laser etc.). The resulting RF signal of the (adequately fast) photodiode is a priori synchronized with the laser excitation. The RF signal can be used analogously with the RF signal of a synthesizer. That is, the phase shifter / amplifier PA unit is made available at the corresponding input (by way of an RF line). The rest of the process is analogous to realization 1. What is advantageous here is, on the one hand, the use of a pulse-shaped light source and the associated improvement of the signal-to-noise ratio in a lifetime contrast, generated thereby, as compared to the sinusoidal modulation of the laser excitation in realization 1 and, on the other hand, the omission of a synthesizer for generating the RF frequency. The fluorescence is detected after a 1 photon excitation (linear in the excitation intensity).

1.2 Two Photon Confocal Lifetime Imaging, Homodyne

The object of this chapter is the combination of 2 photon microscopy with the homodyne detection technique for lifetime imaging.

The combination of time resolution and 2 photon microscopy was already demonstrated by Gratton with the socalled heterodyne technique [So]. In the heterodyne technique an fs laser is used for fluorescence excitation in a 2 photon microscope; and the PMT detector is driven at a slightly different modulation frequency. The typical repetition rate of fs Ti:saphire lasers is in the 80 MHz range. In [So] a frequency of 80 MHz + 25 kHz is used for amplification modulation of the PMT detector. The consequence is a beat. This beat is digitized with an ADC [= analog to digital conversion] card and scanned. Thus, it is possible to detect pixel by pixel the phase shift from excitation to fluorescence (heterodyne method).

In the design presented here, the phase shift is determined, compared to [So], not pixel by pixel, but rather image by image. That is, first one image is captured during a relative phase shift $\varphi 1$ and stored. In at least one other step, an image is generated during another different relative phase $\varphi 2$ (homodyne method).

The diverse images for presenting the contrast are calculated in the same manner as in Chapter 1.1.

Procedure

A dyed or self-fluorescing sample is excited so as to emit fluorescence by way of two photon absorption. The pulsed laser can be, for example, a Ti:saphire laser (also sums with double or triple frequency or a difference with mixed frequency, etc.), a ps laser diode or a laser, which is modulated, for example, by means of an AOM [= acousto-optical modulator] or EOM [= electro-optical modulator]. In 1.1. the physical process is a one photon excitation; in 1.2 it is two photon excitation (or in general multiphoton excitation). To excite the fluorescence in the realization according to Figure 1, an fs Ti:saphire laser is used. The repetition rate of the fs laser is approximately 80 MHz (NLO - LSM [= non-linear optics laser scanning microscope], fluorescence detection method).

For detection, a modulatable PMT detector from Hamamatsu (H6573) is used. The sinusoidal modulation frequency is generated from a photodiode signal of the laser (fs Mira, Coherent Corporation) by way of an ECL [= emitter coupled logic] logic circuit and amplified to an average power of 15.W at 50W by means of an RF amplifier, integrated into the PA phase shifter / amplifier, and is available at the output "RF out" of the PA. The phase of the generated sine (and thus the amplified sine) can be adjusted to approximately 1° (analogous to Chapter 1.1). The electronic jitter is < 100 ps ((Figure 1), phase shifter / RF amplifier). With the amplified sine wave at the frequency, corresponding to the laser repetition rate ($f_{rep} = f_{mod}$), the amplification of the PMT detector is modulated. To this end, the amplified RF frequency is made available to the input of the PMT module, called "RF modulation in" in Figure 1. The current at the output of the PMT detector is smoothed with a passive LP filter or is integrated by a different method by way of the integration circuit. The resulting DC signal is made available again to the detection unit of an LRM. The rest of the procedure for gathering and evaluating the data is analogous to that described in Chapter 1.1.

The distinction with respect to 1.1. lies in the fact that in one case a two photon (NLO) LSM is combined with the lifetime imaging. That is, the fluorescence excitation occurs with an fs or ps pulse laser. Thus, another significant difference is the type of fluorescence excitation (here two or generally multiphotons (> 2) excitation).

1.3 Arrangement for Phase Sensitive Fluorescence Detection in a Laser Scanning Microscope

For two or multiphoton excitation, it is possible to use more efficient detection units due to the depth discriminated excitation. They are described in the literature under the collective term of non-descanned detection.

This detection is shown in Figure 2 as a schematic drawing, which is attached to the scan lens SL and the scanner of Figure 1 and depicts a microscope beam path, with the sample P, the lens OL, the beam splitter ST for coupling in the

the illumination / coupling out the radiation, coming from the sample, as well as a first tube lens TL1.

By way of a second tube lens TL2, another modulatable PMT is used for direct detection in a detection beam path DE, that is, not by way of a scan beam path.

This PMT is connected to the control unit ST in Figure 1 and by way of said control unit to other units in Figure 1.

A significant drawback with these detection units is generally their higher sensitivity to room light. Since the room light is generally not or differently modulated from excitation light, the use of phase sensitive detection units for suppressing the room light is logical. Thus, there is no need for an expensive encapsulation of the detection unit. The configurations for 1 and 2 or multiphoton excitation have already been described above and are totally transferable to a phase sensitive detector. However, in the phase sensitive detection technique the reference signal (excitation light) and the measurement signal (fluorescence signal) have the same modulation. For most of the TiSa laser systems that are used, this modulation is 80 MHz. Thus, homodyne detection takes place. For phase sensitive detection, both signals are multiplied together in a multiplier with a fixed variable phase relation. In an advantageous arrangement the first dynode of a modulatable photomultiplier (H6573) functions as the multiplier.

The reference signal is generated from the above described phase shifter / RF amplifier. The clock for the phase shifter is the photodiode signal of the pulse laser. In addition, the repetition rate, predetermined by means of the pulse laser, can be expanded to a variable beat frequency range by "beating" the laser light with an optical modulator (AOM or EOM, see Figure 1). The beat frequency is provided by means of a synthesizer with variable phase, which is controlled by means of trigger signals of the excitation light source (see Figure 1). The phase relation between the reference signal and the measurement signal is adjusted in such a manner that the phase shift is zero. Thus, the variable is demodulated. In this manner a DC or low frequency modulated measurement signal is obtained. However, disturbances, like room light, are highly modulated

after the multiplier (like the reference signal approx. 80 MHz). Due to the downstream low pass filter the highly modulated portions (disturbances) are filtered out and thus suppressed. An example of the low pass filter is the detection electronics of a conventional LSM.

The advantage of the described arrangement lies in the fact that the electronics on the detection side has to be designed only for low frequency signals, since the demodulation takes place in the photomultiplier.